Antiandrogenic Pesticides Disrupt Sexual Characteristics in the Adult Male Guppy (*Poecilia reticulata*)

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Environmental contaminants have been identified as endocrine disruptors through their antiandrogenic activity. Thus, as androgen receptor antagonists, the fungicide vinclozolin and the principal DDT metabolite *p,p′*-DDE have been demonstrated to induce demasculinization in rats. Whether this is also the case in fish remains to be demonstrated. For a period of 30 days, groups of adult male guppies were exposed to vinclozolin, *p,p′*-DDE, or the therapeutic antiandrogen flutamide (used as positive control) applied to the fodder at concentrations between 0.1 and 100 µg/g fodder. Subsequently, sexual characteristics of relevance to the male reproductive capacity were measured and compared with untreated control fish. All three chemicals caused profound alterations at increasing levels of biological organization, even in these fully matured males. At the cellular level, the three compounds induced a significant reduction in the number of ejaculated sperm cells. At the organ level, the sexually attractive orange-yellow coloration was reduced in area and discolored, and treated fish also had smaller testes. Further, at the organismal level, computer-aided behavior analyses demonstrated a severe disruption in male courtship behavior. We conclude that this demasculinization is consistent with an antiandrogenic action of vinclozolin and *p,p′*-DDE and is likely to compromise reproductive capability in this fish. *Key words*: antiandrogenic effects, courtship behavior, endocrine disruptor, flutamide, guppies, *p,p′*-DDE, *Poecilia reticulata*, sexual characteristics, vinclozolin. *Environ Health Perspect* 109:1063–1070 (2001). [Online 28 September 2001] *http://ehpnet1.niehs.nih.gov/docs/2001/109p1063-1070baatrup/abstract.html*

It is well documented that several chemicals from agricultural, industrial, and household sources possess endocrine-disrupting properties, which potentially pose a threat to human and wildlife reproduction (*1,2*). Most work has focused on the adverse effects of estrogenic substances (*3–5*). The discovery that certain environmental contaminants possess antiandrogenic properties (i.e., disrupt the normal function of the male sex hormones) has added to the endocrine disruption debate (*6,7*). The most intensively studied environmental antiandrogens are the dicarboximide fungicide vinclozolin and the insecticide DDT metabolite *p,p′*-DDE (*8–11*).

Extensive studies have demonstrated that vinclozolin and *p,p′*-DDE interfere with the action of androgens in developing, pubertal, and adult male rats (*10–15*). Exposure to vinclozolin and *p,p′*-DDE during the critical period of sexual differentiation results in sexual abnormalities expressed later in the adult male rat, including reduced anogenital distance, retained nipples, reduced sex accessory gland weights, urogenital malformations, and reduced fertility (*8,10,14,16–18*). With the same molecular mechanism and with almost the same potency as the classical antiandrogenic drug flutamide, both *p,p′*-DDE and the two primary vinclozolin metabolites, M1 and M2, bind the androgen receptor (AR) and act as antagonists by preventing transcription of androgen-dependent genes (*9,11,13,18*). Androgen-induced gene products play a key role in the development and maintenance of male sexual functions, including courtship behavior (*19*) and spermatogenesis (*20*).

The potential threat of environmental antiandrogens to fish and wildlife has been addressed by Monosson et al. (*21*). Although the authors noted that the antiandrogenic activity of *p,p′*-DDE is unknown in nonmammalian species, they suggested that this property may have contributed to the reproductive abnormalities in the American alligators in Lake Apopka (*22,23*) and the near absence of male bloaters in Lake Michigan in the late 1960s.

Androgen receptors have been characterized in a few fish species. Sperry and Thomas (*24,25*) identified two distinct androgen receptors, AR1 and AR2, in brain and gonadal tissues of kelp bass (*Paralabrax clathratus*) and Atlantic croaker (*Micropogonias undulatus*) with different tissue distributions and distinct steroid and xenobiotic-binding specificities. AR1 was found to bind only testosterone with high affinity, but AR2 bound a broader range of natural androgens and antiandrogens, including *p,p′*-DDE and the vinclozolin metabolites M1 and M2. In particular, M2 binds AR2 in both testicular and ovarian tissue with an affinity nearly identical to the AR in rats. Wells and Van der Kraak (*26*) found a single class of high-affinity, low-capacity AR in rainbow trout (*Oncorhynchus mykiss*) brains and in ovaries, testes, and brains of goldfish (*Carassius auratus*). This study suggested a relatively high affinity between *p,p′*-DDE and the goldfish testes AR, whereas *p,p′*-DDE, M1, and M2 showed no significant competition for the AR in any of the remaining tissues tested in the two fish. Likewise, vinclozolin, M1, and M2 failed to compete for high-affinity testosterone binding sites (putative androgen receptors) in the fathead minnow, *Pimephales promelas* (*27*). Accordingly, as pointed out by Sperry and Thomas (*25*), multiple androgen receptor subtypes may be present throughout teleost species and target tissues, with differential affinities to natural androgens and different susceptibilities to xenobiotic interference.

Endocrine-disrupting chemicals (EDCs) are believed to propagate their initial molecular interactions to higher level effects in the endocrine system and reproductive organs, ultimately resulting in an impaired reproductive capability. Thus, disruption of hormonal functions can be expressed at various levels of the vertebrate endocrine system (*28*). Molecular markers (e.g., vitellogenin synthesis and AR binding studies) can be highly sensitive to demonstrate the presence of EDCs in the environment, but the vertebrate endocrine system is so complex that it is impossible to predict higher level effects solely from events at the receptor level. For that purpose it is necessary to identify end points that are more directly related to the reproductive fitness of the individual and preferably with links to population-level effects. We have addressed this objective in a series of laboratory experiments for the purpose of studying the effects of EDCs on selected sexual characteristics in the guppy (*Poecilia reticulata*).

The guppy was chosen as an experimental animal because it is a viviparous fish which breeds year round and has a short reproductive period (*29*). The adult male has a bright orange coloration and performs a distinct courtship behavior. His anal fin is developed into a copulatory organ (the gonopodium) for internal fertilization, and ejaculates of sperm can be evacuated for sperm counting

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without harming the fish (*30,31*). The hormonal pathways controlling the expression of the male sexual characteristics are not fully understood, but sperm production, body coloration, and courtship behavior are known to be regulated by androgens (*32–34*). Hence, the male guppy offers a suite of sexual characteristics that are both accessible for quantification and of relevance for the study of EDCs on reproduction. In this study, adult male guppies were exposed for 30 days to vinclozolin, *p,p′*-DDE, and the therapeutic antiandrogen flutamide, administered in the food. Subsequently, we assessed the effects of these three chemicals on the number of ejaculated sperm cells at the cellular level, body coloration, length of gonopodium (copulatory organ), and relative gonadal weight at the organ level, and finally courtship behavior at the organismal level. Previous studies have demonstrated that most of these end points are altered in adult male guppies exposed to the natural estrogen 17β-estradiol and the xenoestrogen 4-*tert*-octylphenol (*31,35*).

Material and Methods

Animals and experimental conditions. The fish used in this study were healthy, wild-type guppies (*Poecilia reticulata*) imported from Colombia and bred through several generations in 500-L stainless-steel tanks at 25 ± 2°C and a daily 12-hr simulated daylight illumination. These stock aquaria received fully aerated water from a reverse osmosis system (RO-water), which was mixed with local tap water (9:1) and adjusted with NaCl to give a conductivity of 600 µS/cm and a pH of 7.0 ± 0.3. Half of the water in the aquaria was renewed weekly. The guppies were fed daily with freshly hatched *Arthemia sp.* and commercial flake food (TetraMinRubin and TetraMin, Tetra Werke, Melle, Germany).

A total of 260 adult males were chosen randomly from the stock aquarium and divided into 10 experimental groups and 3 control groups. Each group was transferred to a 16-L seamless glass aquarium (Struers Kebo Laboratory, Copenhagen, Denmark) filled with 4 L of RO-water and 4 L of water from the culture tank. During the experimental period of 30 days, the water was constantly circulated through a natural filter of aquarium gravel. Daily, feces were removed and clean RO-water was added to 8 L. To eliminate the risk of leached EDCs, no plastic materials or plants were used in any aquaria and plumbing.

The fish were exposed for 30 days through their food to one of the three antiandrogens: the dicarboximid fungicide vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione], *p,p′*-DDE (*p,p′*-1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene (both chemicals from Riedel-de-Haën AG, Seelze, Germany), and the commercial antiandrogen flutamide (4′-nitro-3′-trifluoromethylisobutyranilide; Sigma Chemicals, St. Louis, MO, USA). Flutamide is a specific inhibitor of the androgen receptor and was therefore used as the positive control of antiandrogenic effects. All three chemicals were dissolved in acetone to adequate concentrations, mixed thoroughly with the commercial TetraMin fish fodder and left for 24 hr in a fume cupboard for the evacuation of acetone. This resulted in fodder contaminated with 1.0, 10.0, and 100.0 µg vinclozolin or flutamide per milligram fodder and 0.1, 1.0 or 10.0 µg *p,p′*-DDE per milligram fodder. The remaining three control groups received food that was mixed with acetone only. Each group of 20 fish was fed daily with 40 mg fodder, corresponding to 0.2, 2, 20, or 200 µg chemical per fish. Assuming an equal consumption of food by the fish and that the average weight of an adult male guppy is 130 mg, the fish at the three application rates of vinclozolin or flutamide were dosed with 15, 150, and 1,500 µg chemical/g fish and fish were dosed with *p,p′*-DDE at 1.5, 15, 150 µg/g fish.

After exposure, we measured male sexual characteristics of importance to guppy reproduction and expected targets of antiandrogenic action at increasing levels of biological organization. The number of ejaculated sperm cells were measured at the cellular level, body coloration, length of gonopodium (copulatory organ), and testis size (gonodosomatic index) at the organ level, and courtship behavior at the organismal level.

Sperm count. Immediately after behavior analysis, the male was lightly anesthetized in ethyl-4-aminobenzoate (Sigma) and placed on a glass plate under an Olympus SZ 40 dissection microscope mounted with a circular illumination of polarized light and a JVC TK-1070E color video camera (Victor Company of Japan LTD, Tokyo, Japan). The gonopodium was swung forward and a 32-bit 1,024 × 1,024 pixel digital image of the fish's left side was captured by a VISTA frame grabber (TRUEVISION, Santa Clara, CA, USA) and stored on disk for later measurements of gonopodial length and coloration. Sperm cells were stripped from the male guppy by gently stroking the abdomen with a small metal rod toward the gonopodium, thereby evacuating an ejaculate on the glass plate. The guppy ejaculate consists of numerous spermatozeugmata (clusters of sperm cells), which were collected with a Finn-pipette and transferred to 90 µL of a 0.125 mM NaCl and 5.0 mM $CaCl_2$ solution to aid the breakdown of the spermatozeugmata. The pipette was filled and emptied 30–40 times to ensured the final disintegration of the spermatozeugmata. Samples of the sperm cell suspension were then transferred to an improved Neubauer chamber hemacytometer (Paul Marienfeld, Bad Mergentheim, Germany) and, after 10 min retention in a humid chamber, counted using the general guidelines for human sperm (*39*). This method gives a measure of the total number of sperm cells in an ejaculate. This method has high reproducibility in individual guppies over time. Toft and Baatrup (*31*) showed, using this method, that the sperm count in uncontaminated guppies remained constant when sampled at time 0, day 30, and day 90.

Gonadosomatic, coloration, and gonopodial indices. The fish was killed in ethyl-4-aminobenzoate and fixed in Lilly's formalin solution. We determined and calculated the wet weights of whole body and testis and calculated the gonadosomatic index (GSI) as the gonadal weight as percentage of the whole-body weight.

The total area of the orange-colored spots was measured in the digital image of the fish and related to the whole body area (fins excluded) as the coloration index. Hereafter, the length of the gonopodium was measured and related to the length of the fish as the gonopodial index. Digital image analyses were performed using GIPS software (Image House, Copenhagen, Denmark).

Male courtship behavior. Sexually mature guppies perform courtship behavior almost continuously during the light hours, all year round. Guppy sexual behavior has been described thoroughly (*36,37*). Briefly, the male places himself in front of the female and stays within her field of view (posturing behavior). From this position he performs the sexual display toward the female known as sigmoid display, where his body assumes the shape of an "S" or "C" (hence the name of this behavior), and vibrates while he swims sideways displaying his sexually attractive orange-yellow coloration. He either moves along the length of the female to come into position for a copulation, or he moves away from the female, remaining in her field of view to entice her to follow. These behavioral maneuvers are so stereotyped and performed so frequently (about 1/min), even in a laboratory setting, that the male guppy's courtship behavior lends itself to quantification. This makes the guppy and its sexual behavior a suitable biomarker of endocrine disruption (*35*).

Analysis of courtship behavior. Guppy courtship behavior was measured automatically using the newly developed computer-aided DISPLAY vision system (Institute of Biological Sciences, University of Aarhus, Denmark), which records and analyzes complex behavior patterns in fish.

After exposure, each male was paired with a 4-month-old, nonreceptive female in a sand-blown 20 × 15 cm aquarium containing 1.8 L of 25°C water (water depth, 10 cm) placed on a sheet of glass 50 cm above diffusely-lit white paper. We used nonreceptive females to preclude copulations and hence to ensure constant female responses toward all males. The entire setup was enclosed in a metal frame covered with a blackout curtain. When viewed from above, this arrangement resulted in clear silhouettes of the two fish, where the male was easily distinguishable from the much larger female (Figure 1). The pairs were left undisturbed for 5 min, after which the scenario was recorded for 10 min.

A CV-M10 progressive scan (non-interlaced) CCD camera (JAI, Inc., Copenhagen, Denmark) mounted 50 cm above the aquarium displayed an image of the aquarium on a monitor. Simultaneously, the analogue video signal from the camera was digitized by a DT3155 frame grabber (Data Translation, Inc., Marlboro, MA, USA) into a 768 × 576-pixel digital image, giving a 0.25-mm spatial resolution of the visual field. The frame grabber was interfaced with a 300-MHZ Pentium II personal computer.

Prior to recording, the interior of the aquarium was framed by a software window (region of interest), and appropriate size and gray-level ranges corresponding to the fish silhouettes were likewise set in the software. These criteria were used for the conversion of each 8-bit gray-scale image into a binary (1-bit) image. Thus, all pixel assemblages fulfilling both size and gray-level criteria (fish silhouettes) were assigned the value 1, while the remaining pixels in the image were given the value 0. This new binary image was stored in a frame file on disk for subsequent analysis. During recording, an image was captured and processed approximately every 1/12 sec, so each 10-min frame file contained about 7,200 binary frames (occupying only about 6 MB disk space).

The frame files, containing the time-series of fish contours, were subsequently analyzed by the DISPLAY program. An exhaustive description of this software is beyond the scope of this paper, but the most important steps in the characterization and quantification of the complex courtship behavior are outlined below.

First, the position and orientation of the two fish within the digital image (global coordinate system) must be established in each frame. The two oblong pixel assemblages, representing the fish silhouettes, are converted into two small coordinate systems by determining their principal axes. The axes can be found by computing the eigenvectors (*38*) for the position vectors of the pixels. The mean pixel position vector is the origin of the coordinate system spanned by the eigenvectors. The eigenvectors constitute a unitary transformation which enables transformation of other coordinate systems into a particular coordinate system, such as the other fish's coordinate system or the global coordinate system. Thus, all angle and distance measures can easily be computed once the principal axes of the pixel masses are determined. In order to associate a directed coordinate system to the pixel mass, the number of object-pixels inside two circles of equal radius are counted two standard deviations from the origin along the *y*-axis in both directions. The direction with the largest pixel count gives the direction of the fish's head. Furthermore, object shape measures can be computed by dividing the object pixel mass into parts. In this case, the parts are simply positive and negative (above and below the *x*-axis) position vectors of the object pixels. Each part is subsequently treated as a new object, and the angle between the new *y*-axes provides a measure of the fish curvature (Figure 1C).

For each frame it is now possible to determine the position and orientation of each fish relative to the other, the distance between them, and the curvature of the male. Further, frame-to-frame comparisons enable calculation of speed and direction (relative to the body's longitudinal axis) of fish movements. The composite courtship behavior of the guppy, including posturing behavior and sigmoid displays, can be broken down into its constituting elements, including the mutual position/orientation of the two fish and their movement patterns. The following parameters were extracted from each of the approximately 7,200 frames in the frame file: *a*) position of female relative to the male measured as the angle (0–180°) between the male's *y*-axis and a line between the origins of the two fish's coordinate systems (centroids; ∡ε in Figure 1A); *b*) position of male relative to the female measured as the angle (0–180°) between the female's *y*-axis and a line between the centroids of the two fish (∡δ in Figure 1A); *c*) distance between the centroids of the two fish (*a* in Figure 1A); *d*) male swimming speed defined as the frame-to-frame displacement of his centroid divided by the time between successive frames (*v* in Figure 1B); *e*) male angular displacement measured as the angle (0–180°) between the male *y*-axis and the position vector of his centroid in the next frame (∡φ in Figure 1B), where high values indicate sideways and backward swimming; *f*) male lateral velocity calculated as the magnitude of the male velocity component perpendicular to the *y*-axis in the preceding frame (*u* in Figure 1B), signifying the intensity of sideways swimming; and *g*) male curvature measured as the angle between the *y*-axes of head region and the tail region, respectively (∡ω in Figure 1C).

The frame-to-frame measurements of the seven parameters were subsequently used to identify periods with posturing behavior and sigmoid displays within the entire 10-min frame file. This was done by assigning a range of pass values (search criterion) to each parameter. Basically, a specific behavior is recognized when all search criteria are fulfilled simultaneously. Thus, before analyzing the frame files, the appropriate combination of search criteria describing the specific behavior is set once for all. This is done by repeatedly refining and verifying the search

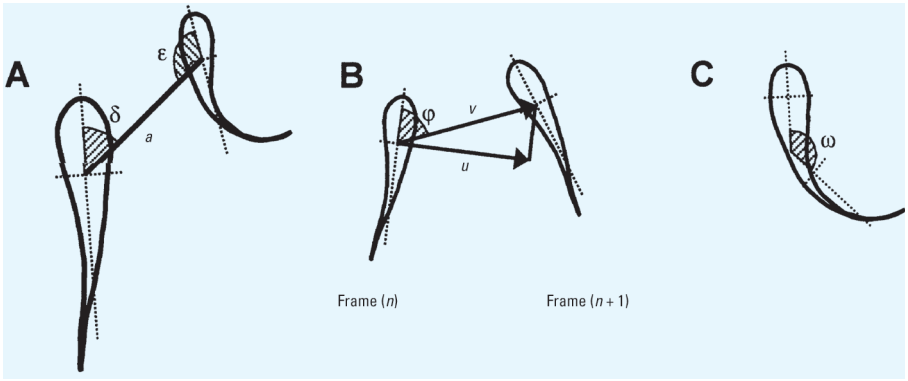

Figure 1. Composite courtship behavior of the male guppy automatically quantified by the DISPLAY vision system using seven descriptive components. Abbreviations: *a*, distance between female and male; *u*, male lateral velocity measured as the velocity component perpendicular to the longitudinal axis of the male; *v*, male swimming velocity; ∡δ, angular position of the male relative to the female's longitudinal axis; ∡ε, angular position of the female relative to the male's longitudinal axis; ∡φ, male angular displacement relative to his longitudinal axis; ∡ω, male curvature measured as angle between the y-axes of head region and tail region. (*A*) The two oblong pixel assemblages representing the male and female silhouettes from the digital image were converted into directed coordinate systems, which enabled the calculation of position and orientation of each fish relative to the other and the distance between them. (*B*) Frame-to-frame comparisons enabled calculations of speed and direction (relative to the body's longitudinal axis) of fish movements. (*C*) The body curvature was measured as the angle between the two y-axes of coordinate systems aligned with the head and tail regions, respectively.

criteria until the software's interpretation of the behavior in all situations agrees with this particular behavior. This manipulation is easily performed by combining a graphical user interface for entering search criteria with a real-time replay facility in the DISPLAY program.

The measurement of the male's posturing behavior (i.e., the time he spent in front of the female introducing the next sigmoid display) involved three search criteria. First, the male must be in front of the female (pass values of ∡δ in Figure 1A set to $0 < \delta < 90°$). Second, the male must at least partly face the female (∡ε criterion in Figure 1A set to $0 < \varepsilon < 60°$). Note that all angles are presented without signs since there is no distinction between the right side and left side of the fish. Finally, the two fish must be within a distance of a few centimeters (*a* in Figure 1A set to < 60 mm).

The guppy sigmoid display is a much more complex behavior. First, the combination of involved parameters and their ranges of pass values changes during the course of the display. Accordingly, a positive identification of the entire display by the software requires temporal adjustments of the search criteria. This was achieved by associating a timer to each search criterion, engaging and disengaging its function. The onset of the display is characterized by the male being within the anterior part of the female's field of view for at least 0.2 sec ($0 < \delta < 90°$; time out 0.2 sec) at a distance of at least 22 mm ($a > 22$ mm) exposing the side of his body ($\varepsilon > 68°$), which is locked in a distinct curvature for at least 0.5 sec ($\omega > 20°$; time out 0.5 sec). After this initial phase the rules are changed allowing the male to move within the female's entire field of view ($0 < \delta < 140°$), but with his body still locked in a curvature ($\omega > 5°$) with the convex side continuously facing her ($\varepsilon > 68°$). Display termination is registered when one or more of these criteria are no longer fulfilled.

The frame files of all treated and control fish were analyzed by the DISPLAY software. The complete analysis of a 10-min frame file takes about 25 sec with a 300 MHz Pentium II computer. The time devoted to positioning behavior, number, and duration of the sigmoid displays and the average values of the measured parameters were saved in a data file for subsequent statistical analysis.

Statistical analyses. Where necessary, data sets were transformed to comply with the normality and variance homogeneity requirements for analysis of variance (ANOVA) testing to compare means among the treatment groups. Subsequently, Dunnett's test for multiple comparisons was used to determine whether treatment means were significantly different ($p < 0.05$) from the control group. All statistical tests were performed with SPSS software (SPSS for Windows, release 9.0; SPSS Inc., Chicago, Ill, USA).

Results

In the group fed the highest concentration of vinclozolin (100 µg/mg), 15% of the fish died during the 30 days of treatment. The corresponding mortalities with the highest concentrations of DDE (10 µg/mg) and flutamide (100 µg/mg) were 70% and 35%, respectively. Because the chemicals were obviously toxic at these application rates, these three groups were excluded from further evaluation of antiandrogenic effects. In contrast, at the 10 and 100 times lower application rates, none of the fish displayed obvious toxic responses such as body darkening or changed swimming activity. The few fish that were lost in these groups died solely because of incorrect handling.

All three chemicals caused pronounced effects on the adult male's sperm count, body coloration, testes size, and courtship behavior. Below, the effects on these sexual characteristics are presented in the order they were measured.

Sperm count. The provoked ejaculate from control fish contained an average 5 million sperm cells. All antiandrogen treatments reduced this number, except in the group fed *p,p′*-DDE at 0.1 µg/mg, where the sperm count actually exceeded that of control fish (Figure 2B). The lowest sperm count, at 1.6 million sperm cells, was measured in the 10 µg/mg flutamide group. The ejaculates of the remaining groups contained 3–4 million sperm cells.

Coloration and gonopodial indices. The demasculinizing effects of the antiandrogens also influenced the area and color intensity of the male orange coloration. In the control group, an average 12% of the body surface (coloration index) was coved with orange spots, while this percentage was lower in the treated groups (Figure 2C). Statistically, the reduction in coloration index was only significantly different from the control fish in the group treated with vinclozolin at 1 µg/mg. Even with the naked eye it was obvious that the treatments also caused discoloring of these sexually attractive spots. Measurements of the red, green, and blue color components in the digital images demonstrated that this fading was primarily caused by a significant brightening of the blue component in all treated groups, with the exception of the low dose *p,p′*-DDE group (data not shown).

The length of the gonopodium relative to the length of the fish (gonopodial index) was unaffected by the antiandrogens, as was the size of the fish.

Gonadosomatic index. The weight of the testis relative to the body weight (gonadosomatic index) was significantly lower in the fish exposed to antiandrogens with the exception of the group treated with the low *p,p′*-DDE dose (Figure 2D). In the control

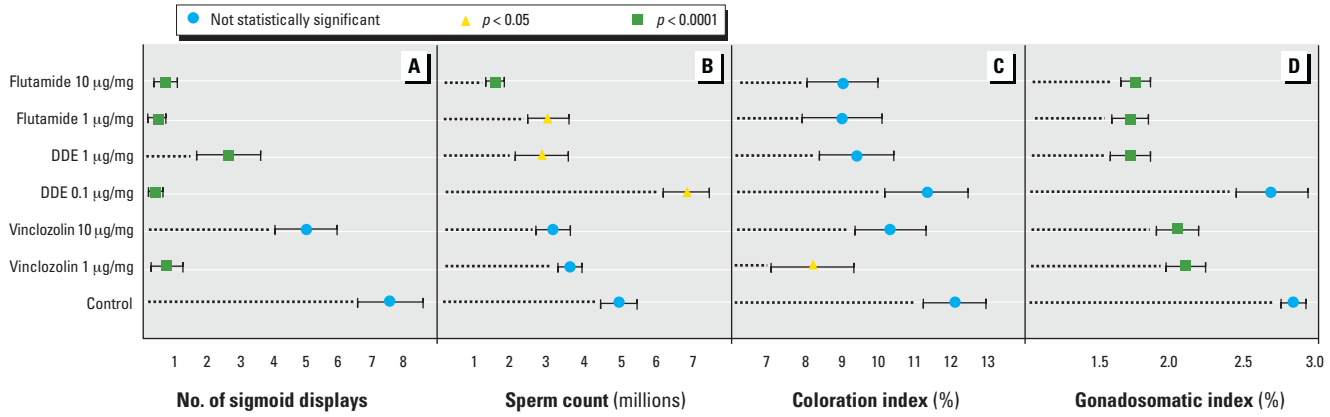

Figure 2. The effects of a 30-day exposure to vinclozolin, *p,p′*-DDE, and flutamide on four sexual characteristics of the adult male guppy. (*A*) Number of sigmoid displays during the 10-min recording period. (*B*) Number of sperm cells in a provoked ejaculate. (*C*) Area of orange coloration as percentage of body area (coloration index). (*D*) Weight of testis as a percentage of whole-body weight (gonadosomatic index). Statistical differences between the control group and the treated groups were tested with one-way ANOVA followed by Dunnett's post-hoc multiple comparisons test.

group, the testes made up about 2.8% of the body weight, whereas GSI values between 1.7 and 2.0% characterized fish treated with the three chemicals.

Courtship behavior. Of the seven behavioral elements (Figure 1) measured by the automated vision system, four were used in combinations to quantify the two most important behavioral patterns in the courtship behavior—namely, the posturing behavior and the sigmoid display. The accuracy by which the vision system identified these two composite behavior patterns was assessed by replaying the frame sequences. All situations with posturing behavior were correctly quantified by the system, and of 464 sigmoid displays identified in the 145 recordings, only 24 cases were considered questionable by two independent observers and therefore excluded from further analysis.

The effects of vinclozolin, *p,p′*-DDE, and flutamide on each of the seven behavioral elements are presented as average values in Table 1. Considered individually, two behavioral components of the sexually active male guppies were particularly affected by the three compounds. The males from the treated groups were less oriented toward the female (∡ε in Table 1) and swam less sideways, both as regards swimming direction (∡φ in Table 1) and sideways swimming velocity (*u* in Table 1). The male's efforts to face the female were most strongly restrained by vinclozolin at 1.0 µg/mg fodder, whereas *p,p′*-DDE most effectively impeded the sideways swimming activity. Surprisingly, some of the behavioral elements displayed a negative or neutral dose–response relationship. For instance, the inhibition of male orientation toward the female and his sideways swimming was more pronounced in the group that was treated with 1 µg vinclozolin/mg fodder than in the group treated with the 10 times higher concentration. The same two behavioral components were affected to the same degree by the two DDE concentrations. The remaining elements were less influenced by the antiandrogenic treatments, including the male's position relative to the female (∡δ in Table 1), his body curvature (∡ω in Table 1), and the distance between the two fish (a in Table 1). It is worth noting that there was no statistical difference in the general swimming velocity (*v* in Table 1) between the males in the treated groups and the control group. Significant differences in average swimming velocity could indicate a general toxic effect of the chemicals.

The two composite patterns in the male guppy's courtship behavior, the posturing behavior and the sigmoid display, were more strongly affected by the three antiandrogens than any of the separate constituent components. In all cases, the chemicals weakened the male's sexual activity. With the face-to-face posturing behavior, the male tried to attract the female's attention before he performed the sigmoid display. The influence of the three antiandrogens on the duration of posturing behavior within the 10 min of recording is shown in Figure 3. The males from the control group spent on average 130 sec in posturing behavior, corresponding to 22% of the observation period. The most serious inhibition of this behavior was found in the group fed vinclozolin at 1 µg/mg fodder, where the males on average fulfilled the criteria of posturing behavior for only 40 sec, or 7% of the recording period. In comparison, the group treated with flutamide at the same concentration spent about 15% of the time on posturing. The composite behaviors also demonstrated neutral or negative dose–response relationships. Hence, in the group treated with vinclozolin concentration at 10 µg/mg fodder, the posturing behavior was unaffected, whereas flutamide at this concentration inhibited this behavior by 51% relative to the controls. Correspondingly, *p,p′*-DDE restrained the posturing behavior by 20% and 43% at 0.1 and 1.0 µg/mg, respectively, when compared with the control group.

Although the sigmoid display is the most conspicuous movement pattern in the male guppy's courtship behavior, it makes up only a small part of the courtship temporally. The total duration of the male's mating behavior, including both posturing behavior and sigmoid displays, is presented in Figure 3. As a prelude to the copulation attempt itself, the sigmoid display is the culmination of the courtship behavior. The number of sigmoid displays is therefore a suitable measure of the male's mating ardor. Figure 2A demonstrates that males from the control group performed on average about eight sigmoid displays per 10 min observation period, whereas this behavior only rarely occurred in the groups treated with flutamide or the lowest concentrations of vinclozolin (1.0 µg/mg) and *p,p′*-DDE (0.1 µg/mg). This component in the courtship behavior also demonstrated a negative dose–response relationship, with much less inhibition at the high application rates of the two pesticides.

Discussion

Oral administration of either vinclozolin, *p,p′*-DDE, or flutamide clearly altered the sexual characteristics of the adult male guppy. After only 30 days of exposure, the orange display coloration was reduced in both area and color

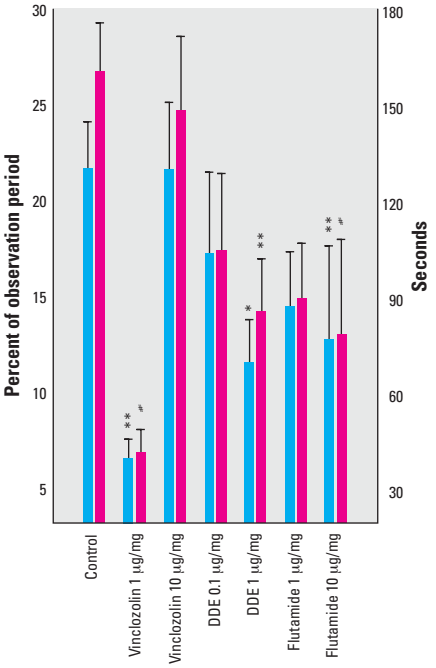

Figure 3. The three antiandrogens, vinclozolin, *p,p′*-DDE, and flutamide, reduced the time allocated to courtship behavior in the adult male guppy. Blue bars indicate the average duration of posturing behavior during the 10-min recording period. Red bars show the total time devoted to both posturing behavior and sigmoid displays.
**p* < 0.05, ***p* < 0 .001, and #*p* < 0.0001 as determined by Dunnett's post-hoc test.

Table 1. The effects of vinclozolin, *p,p′*-DDE, and flutamide on seven components in the courtship behavior of the male guppy.

Treatment (µg/mg fodder)	No.	∡ε (degrees)	∡δ (degrees)	*a* (mm)	*v* (mm/sec)	∡φ (degrees)	*u* (mm/sec)	∡ω (degrees)
Control	(19)	44.1 ± 4.3	84.6 ± 5.8	54.9 ± 3.4	17.4 ± 1.5	69.6 ± 2.8	7.4 ± 0.7	9.4 ± 0.4
Vinclozolin, 1.0	(18)	82.5 ± 4.4#	80.2 ± 4.7	75.2 ± 6.8*	20.4 ± 2.5	46.2 ± 3.0#	4.5 ± 0.6**	8.5 ± 0.6
Vinclozolin, 10.0	(18)	60.7 ± 5.5	75.8 ± 3.2	53.9 ± 3.5	18.8 ± 1.8	59.7 ± 5.3	5.2 ± 0.5**	7.5 ± 0.4
DDE 0.1	(17)	69.2 ± 6.6**	81.5 ± 4.1	58.1 ± 6.5	13.2 ± 2.2	59.6 ± 4.8	3.1 ± 0.6#	6.4 ± 0.6#
DDE 1.0	(16)	68.0 ± 5.3**	75.8 ± 3.5	68.4 ± 6.2	15.3 ± 2.1	51.6 ± 6.0*	3.5 ± 0.4#	7.1 ± 0.6
Flutaminde,1.0	(17)	71.1 ± 5.3**	79.8 ± 3.5	67.6 ± 6.5	16.0 ± 1.9	52.4 ± 3.7**	4.6 ± 0.6**	8.5 ± 0.7
Flutaminde, 10.0	(18)	78.7 ± 5.8#	77.3 ± 4.7	91.8 ± 8.7#	19.6 ± 1.8	39.6 ± 3.4#	4.6 ± 0.5#	7.2 ± 0.3#

Differences between treated groups and control group were tested with one-way ANOVA followed by Dunnett's post-hoc multiple comparisons.
**p* < 0.05; ***p* < 0.001; #*p* < 0.0001.

intensity, the weight of the testis was diminished, the sperm count had fallen, and the courtship behavior was almost extinguished. To our knowledge, the present study provides the first evidence that these pesticides can cause severe reproductive abnormalities in fish.

Two previous studies, involving *p,p′*-DDE and vinclozolin concluded that there was no evidence that these two chemicals act as endocrine disruptors in fish. Carlson et al. (*40*) microinjected embryos of rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) with a number of contaminants, including *p,p′*-DDE. After rearing for 6 months, no treatment-dependent changes in sex ratio, gonadal histology, or steroid production were observed. Similarly, embryonic fathead minnows (*Pimerhales promelas*) were exposed to vinclozolin in the water by Makynen et al. (*27*), who concluded that vinclozolin had no adverse effects with respect to sexual differentiation and reproductive success despite the fact that data showed a 20–60% reduction in fecundity. In contrast, exposure of adult fathead minnows to vinclozolin resulted in increased plasma 17β-estradiol in males and a decline in the gonadosomatic index of females accompanied by a retardation in oocyte development (*27*). Accordingly, the three studies performed till now reached different conclusions regarding the endocrine-disrupting properties of vinclozolin and *p,p′*-DDE. These apparent discrepancies may be explained by differences in chemical concentrations, route of application and time-window of exposure, but more likely reflect real species differences in the sensitivity of the reproductive apparatus to antiandrogenic compounds. Considering that fish display a wide range of reproductive strategies and the growing evidence of interspecies and tissue differences in AR binding specificity, some fish may be more susceptible than others to endocrine disruption by a particular chemical (*21,25,26*).

The three chemicals used in this study caused impairment of the guppy's sexual characteristics in a manner consistent with the effects of antiandrogens. First, the affected sex characteristics are known to be under androgen control in the guppy (*32,41–43*). Thus, Pandey (*32*) blocked the synthesis of sex steroids in adult male guppies by hypophysectomy, which caused a marked regression in the testis, inhibition of spermatogenesis, and a pronounced fading of the orange display coloration. Subsequent treatment of these hypophysectomized males with the androgen methyl testosterone induced partial restoration of coloration and testis morphology and function (*42*). Similarly, it has been shown that the guppy's sexual behavior is under androgen control (*37,41,44*), as is the case with other fish species (*33*). Accordingly, the changes in the sexual characteristics induced by vinclozolin and *p,p′*-DDE closely parallel those evoked by androgen deprivation. Second, it has been thoroughly established that both vinclozolin and *p,p′*-DDE are functional antiandrogens in mammals by blocking the androgen receptor (*9,10,12,18*) in the same way as the therapeutic drug flutamide, which acts purely as an antiandrogen (*45,46*). Collectively, these considerations strongly suggest that vinclozolin and *p,p′*-DDE act as endocrine disruptors in the guppy by antagonizing the androgen receptor. The altered sexual characteristics represent a significant reduction in the expression of the male phenotype, indicating that the reproductive fitness of antiandrogen treated fish was impaired.

An appropriate sexual behavior is prerequisite for mating success in most animals. Unbiased measures of the guppy courtship behavior were obtained using the newly developed vision system, designed to identify complex behavioral patterns in fish. The sexual instinct of the male guppy was seriously compromised by vinclozolin, *p,p′*-DDE, and flutamide, which significantly reduced the time devoted to posturing behavior and almost eliminated sigmoid displays. It has been demonstrated that males with a high sigmoid display frequency are preferred by females (*47,48*) and that male mating success is positively correlated to the intensity of sigmoid displays (*47,49,50*). Also, Matthews et al. (*30*) found a strong correlation between display rate and sperm number, hence providing further evidence of the link between sexual behavior and reproductive capacity in male guppies.

The male orange coloration is similarly thought to signal condition and genetic quality. Several studies have shown that male guppies with the largest and brightest orange spots are favored by females and that these males have a higher mating success (*48,51,52*). Impairment of the male coloration is therefore likely to reduce reproductive fitness. In addition to antiandrogens, discoloration of the orange spots has been reported in response to other chemical and natural stressors, including estrogen (*31,53*), the xenoestrogen octylphenol (*31*), food quality (*50*), and parasites (*54*).

The relationship between sperm count and Darwinian fitness is less clear. Kime (*55*) noted that it is difficult to relate sperm count in fish to population-level effects because the amount of ejaculate necessary for successful fertilization is unknown. However, Warner (*56*) has argued that most male fish release only the minimum amount of sperm that is required for fertilization, so that any decrease in sperm quantity or quality will result in reduced fertility. In this study, the number of sperm cells in the provoked ejaculates of the treated groups was reduced 20–60% when compared with the control group. This reduction in sperm count may be a simple consequence of the diminished testis size in the exposed fish and/or caused by a direct antiandrogenic action of the chemicals on spermatogenesis. Inhibited spermatogenesis in response to antiandrogenic compounds has been reported in a number of vertebrates, including fish (*57*), amphibia (*58*), hamsters (*59*), and humans (*60*). In particular, significant reductions in epididymal sperm counts have been demonstrated in rats treated with *p,p′*-DDE (*61*) and vinclozolin (*18*). In contrast, Moorman et al. (*62*) found a surprising increase in sperm counts in sexually mature rabbits after dermal application of vinclozolin during the peripubertal period. Still, inhibited spermatogenesis appears to be the general rule of antiandrogenic exposure.

The gonopodium was unaffected by exposure to vinclozolin, *p,p′*-DDE, or flutamide. This is in agreement with Pandey (*42*), who found that the morphology of the gonopodium in adults was insensitive to steroid depletion by hypophysectomy and concluded that once morphogenesis of skeletal elements is completed, it becomes independent of the pituitary hormones and androgens. The development of the gonopodium is certainly under androgen control because adult female guppies fed 17α-methyltestosterone developed gonopodia (*44*). Also, a parallel study in our laboratory has demonstrated that significantly smaller gonopodia evolved in guppies treated with the three antiandrogens during juvenile development (*63*).

Overall, the three antiandrogens affected the selected sexual characteristics in the same direction. Identical amounts of fodder were added to all aquaria daily, and all fodder was consumed before feeding the following day. The fish appeared eager to feed in all treatments and throughout the experiment, so we could not detect any possible differences in palatability between chemicals. Considering the molar concentrations of the three chemicals in the fodder (vinclozolin: 3.5 and 35 µmol/g fodder; *p,p′*-DDE: 0.31 and 3.1 µmol/g fodder; flutamide: 3.6 and 36 µmol/g fodder), it appears that the antiandrogenic potencies *in vivo* of vinclozolin and *p,p′*-DDE equaled, and in some cases even exceeded, that of flutamide. However, the lipophility of *p,p′*-DDE (log K_{ow} 6.51) is more than three orders of magnitude greater than flutamide (log K_{ow} 3.35) and vinclozolin (log K_{ow} 3.10). The strong relationship between this factor and the uptake constant (*64*), depuration rate constant (*65*), and bioconcentration factor in fish (*66*) probably

resulted in higher tissue concentrations of *p,p′*-DDE than vinclozolin or flutamide in our experiment. Determination of the relative potencies of these three chemicals will require investigation of chemical concentrations at the target tissues. This difference in lipophility may also explain the noteworthy effect pattern seen with the lowest dose of *p,p′*-DDE. Here, sexual behavior was almost eliminated, but no effects were seen in sperm count, GSI, or display coloration. It is reasonable to expect that *p,p′*-DDE is rapidly partitioned into tissues with a high lipid content, such as the brain, thus affecting behavior before other secondary sex characteristics. However, the response pattern of *p,p′*-DDE is also known to differ from those of vinclozolin and flutamide in the rat (*10,12,18*). Finally, it is possible that these differences are caused by subtle, tissue-dependent differences in AR affinities for antiandrogens as seen in other fish species (*24,25*).

Some of the measured parameters exhibited neutral or negative dose responses (i.e., the higher dose produced a weaker response than the low dose). From a traditional toxicologic viewpoint, this type of bell-shaped dose–response curve is somewhat surprising, although such responses are common in physiologic and in certain hormone studies. A survey of the literature from the last decade revealed nearly 100 titles reporting this type of response in hormone research. Of particular relevance to the present study, Wong et al. (*11*) demonstrated that the M2 metabolite of vinclozolin binds the AR, producing a ligand that can enter the nucleus. The presence of even small quantities of the natural androgen dihydroxytestosterone distorted this ligand, preventing the induction of DNA transcription, making M2 a functional antiandrogen. However, in the absence of dihydroxytestosterone, DNA transcription proceeded and M2 functioned as an androgen analogue, leading the authors to suggest that M2 functions as an androgen at high *in vivo* concentrations.

In conclusion, this study demonstrates that *p,p′*-DDE, vinclozolin, and flutamide caused profound demasculinization of fully matured male guppies, impairing male sexual characteristics from the cellular level to the organismal level after only 30 days of exposure. In a parallel study, Bayley et al. (*63*) measured the same end points in adult male guppies after exposure to the same three chemicals throughout the juvenile period. Similar results were obtained for sperm count and courtship behavior, but the actual size of the testis (GSI) was unaffected by the treatments. In addition, juvenile exposure caused delayed sexual maturation and a skewed sex ratio toward female predominance at adulthood in treated groups.

The impairments of the guppy's sexual characteristics are consistent with an antiandrogenic action of vinclozolin and *p,p′*-DDE. However, it is noteworthy that some of the measured end points, including body coloration and sexual behavior, responded similarly to estrogenic compounds (*31,35*), suggesting that demasculinizing and feminizing endocrine disruptors may have common molecular targets and/or cellular responses. Studies of the interactions between these chemicals and sex steroid receptors are required in the guppy to provide concrete evidence of the mechanisms underlying these effects on the sexual phenotype.

A number of fundamental questions remain unanswered. First, we need to confirm or disprove our assumption that the measured changes in the male sexual characteristics are actually translated into an impaired reproduction. Also, the possible effects of vinclozolin and *p,p′*-DDE on female fertility should be investigated, for instance, by mating exposed males with unexposed females and vice versa. Finally, long-term exposure to environmentally realistic concentrations, involving several generations and all life stages, should be carried out. These studies are currently being performed in our laboratory.

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